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Cholesterol as a Limiting Factor in the Growth of *Mycoplasma pneumoniae*

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Ultracentrifugation was used to separate three commercial lots of bovine serum fraction (BSF) into components designed to contain lipoproteins. Each BSF lot and component was tested for ability to support the growth of three strains of *Mycoplasma pneumoniae*. In general, the level of growth-promoting activity corresponded to the amount of cholesterol present in the BSF or BSF components rather than to the amount or type of lipoprotein. Cholesterol was the limiting nutritional factor of BSF with low growth-promoting activity. The addition of cholesterol and bovine serum albumin to BSF with low activity resulted in growth equal to or greater than that observed for BSF with high growth-promoting activity. When cholesterol was added to agar medium containing BSF of low activity, mycoplasma colonies were greater in number, possessed larger mean diameters, and had centers that were more distinct than those observed when this BSF was used alone. Variability in growth-promoting actions of commercial lots of BSF was eliminated by increasing their cholesterol content to an optimum level. An adjustment of the cholesterol and albumin levels of any serum product used in culture media may provide a simple convenient method to improve growth and isolation of mycoplasmas.

Mycoplasma species contain sterols in their membranes, a characteristic that distinguishes them from other procaryotes. Since they cannot synthesize sterols, serum is often used as an exogenous source of cholesterol. In 1951, Smith and Morton (17) prepared, by ammonium sulfate precipitation, a fraction of bovine serum that contained all the growth-promoting properties of unfractionated serum. This bovine serum fraction (BSF) was later reported (18) to contain alpha, or high-density, lipoprotein (HDL), and it was postulated that mycoplasmas grown in bovine serum obtained cholesterol from HDL.

Slutzky et al. (15, 16) assessed the growth-promoting ability of human lipoproteins. They found greater activity in beta, or low-density, lipoprotein (LDL) than in HDL. Washburn and Somerson (23) obtained similar data with human serum, but results with horse and bovine sera indicated that HDL was more active than LDL. They noted that the most active lipoprotein for each species, i.e., HDL in cattle and horses and LDL in humans, also carried the majority of the serum lipid for that species.

In other studies, Washburn and co-workers (22) used a sequential ultracentrifugation procedure for a density separation of lipoproteins from BSF. In contrast to previous work, the greatest activity of all the components was found in the material designed to contain LDL, even though no intact lipoprotein could be detected by electrophoresis.

The requirement by *Mycoplasma* organisms for cholesterol has been demonstrated by many workers. Razin and Tully (12) determined the minimum amount of cholesterol necessary for the growth of several *Mycoplasma* species in broth by using a cholesterol suspension dissolved in Tween 80. However, on occasion, the preparation of the suspension was troublesome and, in addition, the suspension was toxic for some species. They also reported considerable difficulty in culturing *Mycoplasma pneumoniae* in serum-free broth; this problem was overcome only when a large inoculum was used. Edward (4) determined the minimum cholesterol requirement for several *Mycoplasma* species on agar. The cholesterol was suspended in water, a method which eliminated the problem of toxicity.

In our study, we further fractionated three lots of BSF by ultracentrifugation, and we used *M. pneumoniae* to ascertain the growth-promoting ability of some of the components. Our growth conditions, in which mycoplasmas attach to glass, simplify the quantitation of cultures grown in broth (3, 22). In addition, we used agar medium to confirm the results and to determine the morphological features of the colonies. The differences in the quality of BSF lots was shown to be due to variations in their cholesterol con-

centration. We determined the concentrations of cholesterol that gave maximum growth of *M. pneumoniae* and showed that although cholesterol and albumin could, at least partially, replace BSF, the limiting growth factor was the cholesterol concentration.

MATERIALS AND METHODS

Growth media. The broth medium was serum substitute revision no. 2 (SSR_2) as described by Somerson et al. (20). This medium consists of an autoclaved broth base to which are added several supplements. Included in the base are mycoplasma broth base (BBL Microbiology Systems, Cockeysville, Md.) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (HEPES; Calbiochem-Behring Corp., La Jolla, Calif.). The supplements consisted of Eagle minimum essential medium with glutamine (KC Biologicals, Inc., Lenexa, Kans.), yeast extract, glucose, phenol red, penicillin G, and BSF (PPLO serum fraction, Difco Laboratories, Detroit, Mich.). SSR_2 plates were prepared with PPLO agar plus agar (Difco) to give a final agar concentration of approximately 1.1%. Except for the omission of phenol red, supplements to solid media were the same as those for broth. Each medium ingredient was purchased from one commercial source and consisted of a single lot.

BSF. Four different lots of serum fraction (Difco) were assigned letter designations BSF-A through BSF-D. Most of BSF-A (Lot 591725) had previously been consumed in a nutritional study on BSF (22). We had a limited sample stored and used it for lipoprotein studies only. BSF-B was employed for routine cultivation of mycoplasmas, including adaptation of organisms to SSR_2 and preparation of pools of mycoplasmas. Unless otherwise specified, all BSF and BSF ultracentrifugation fractions were used at a final concentration of 3%.

Medium supplements. Since Fleischman's 20–40 yeast was not available, a yeast extract solution was prepared in our laboratory from powdered brewer's food yeast (supplied by Anheuser-Busch, St. Louis, Mo.). Our modification of the procedure of Hayflick (8) included an initial autoclaving of the 25% (wt/vol) suspension. After cooling, the pH was increased to 8.0 with 10 N NaOH, and the extract was centrifuged in a Sorvall RC2-B for 30 min at 600 rpm in a GS3 rotor. The supernatant was autoclaved, and the flocculant material was allowed to settle. The solids were discarded, and the fluid phase was used as the final product.

In several experiments fraction V, fatty acid-free bovine serum albumin (BSA; Miles Laboratories, Inc., Kankakee, Ill.) served as a protein supplement to the medium. It was prepared as a 10% solution in distilled, demineralized water and sterilized by passage through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). When tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (by C. Findell, Ohio State University), the only major band was that of albumin, although several faint bands of lower molecular weight were visible when the slab gel was dried. There were no lipids detectable by four-directional thin-layer chromatography (11).

Cholesterol (Sigma Chemical Co., St. Louis, Mo.; Merck & Co., Inc., Rahway, N.J.) was sometimes used as a medium supplement. For addition to broth and agar, we prepared a cholesterol suspension in water by the method of Edward and Fitzgerald (5). This procedure involved the addition of a solution of cholesterol in absolute ethanol to water, and it yielded a uniform suspension that was stable for several weeks at 10°C. The amount of ethanol needed to obtain desired cholesterol levels in the medium was not inhibitory to mycoplasmas.

Mycoplasma stock cultures. All *M. pneumoniae* isolates were cultured as confluent layers of organisms on glass (19) and were adapted to SSR_2 medium for several passages before freezing. For the preparation of pools, the glass-adherent mycoplasmas (GAM) were removed from the bottle surface by glass beads and suspended in serum-free SSR_2 containing 15% sucrose before storage at −60°C in 1.2-ml aliquots in 1-dram (ca. 3.7-ml) vials. The species identification was confirmed by immunofluorescence by R. Del Guidice at Frederick Cancer Research Center, Frederick, Md.

We used three *M. pneumoniae* strains of various passage histories and stages of adaptation to growth on artificial media. Both strains CL-8 and 65-2161 were frozen at the relatively low passage levels of 14 and 26, respectively. Strain CL-8 was isolated on medium containing BSF; strain 65-2161 was isolated from a specimen supplied by Children's Hospital, Washington, D.C. Strain FH was originally obtained from the National Institutes of Health and was extensively subcultured on artificial media before freezing at the 336th passage.

Assessment of mycoplasma growth. Each inoculum was prepared by adding the thawed contents of one vial from a frozen pool to a 6-ounce (ca. 180-ml) Brockway prescription bottle containing 25 ml of broth, and the bottle was incubated at 37°C. Strain FH was harvested at 48 h; *M. pneumoniae* strains 65-2161 and CL-8 were harvested at 66 to 72 h. We decanted the culture fluids and used glass beads to suspend the GAM in 2.0 or 2.5 ml of fresh serum-free SSR_2 broth. For broth experiments, we inoculated each 3-ounce (ca. 90-ml) bottle with 0.1 ml of the myocplasma suspension.

Mycoplasmas were grown attached to glass in 3-ounce prescription bottles in triplicate, each bottle containing a total volume of 12 ml of medium. Cultures were incubated for the time period noted above. We assessed growth of the GAM qualitatively by microscopic examination. Characteristics of a well-formed cell layer were high density on glass and uniformity of growth. To determine the amount of GAM protein, we discarded the broth and washed the cell sheet four times with 5-ml volumes of phosphate-buffered saline, pH 7.2. The GAM were removed with glass beads and suspended in 1 to 2 ml of phosphate-buffered saline.

For inoculation of agar plates, the GAM were harvested as described above and diluted in serum-free SSR_2; 0.1 ml of the appropriate dilution of inoculum was spread on the surface of the agar. Plates were incubated aerobically at 37°C.

Protein assays. Protein was measured by the procedure of Lowry et al. (9) or the Coomassie brilliant blue G-250 assay (Bio-Rad Laboratories, Richmond,

Calif.). Results were expressed as micrograms of GAM protein per bottle. The G-250 assay was faster, more convenient, and as reliable as the Lowry method. In addition, many compounds, such as HEPES, are known to interfere with the Lowry assay but not the G-250 assay (1). A micro version of the G-250 assay was performed for most of our experiments. In this method, based on the work of Spector (21), 0.5 ml of the G-250 reagent was combined with 0.1 ml of diluted GAM suspension and placed in glass microcuvettes. All dilutions were made in phosphate-buffered saline. Absorbance was read on a Gilford 240 spectrophotometer at 595 nm. Protein values were determined from a BSA standard curve.

Separation of lipoprotein components. To accommodate large amounts of BSF, we modified the ultracentrifugation procedure of Hatch and Lees (7) for the separation of lipoprotein classes by flotation. A Beckman 60Ti rotor and thick-walled, polycarbonate tubes (25.4 by 89 mm) without caps were used at 54,000 rpm in a Beckman L-2 preparative ultracentrifuge. Salt solutions of increasing densities were added to BSF samples before each of three sequential spins designed to separate the very-low-density lipoproteins, LDL, and HDL. We avoided a preliminary spin in which chylomicrons were separated, and removed this material along with the very-low-density lipoproteins. Because of the increased sample size, we lengthened the duration of the spins to 14, 17, and 34 h; these figures were obtained from several preliminary separation trials.

Immediately after isolation, all ultracentrifugation components were dialyzed for 24 h against 1,000 volumes of a solution containing 0.15 M NaCl, 10^{-3} M ethylenediaminetetraacetic acid, and potassium penicillin G at 500,000 U/liter. The components were refrigerated until needed and were dialyzed again for 3 to 4 h at room temperature against 200 volumes of 0.15 M NaCl just before incorporation into mycoplasma medium. The products isolated from spins designed to contain LDL and HDL are referred to as components 3 and 4. When either component 3 or 4 was added to the medium as a BSF substitute, 0.4% BSA was included. Lipoprotein electrophoresis of BSF-A, -B, and -C and their third and fourth components was performed on agarose strips by the Ohio State University Hospital clinical chemistry laboratory by the method of Noble (10).

Photomicrography. Photomicrography of *M. pneumoniae* on agar was performed with a Nikon phase microscope; micrographs of *M. pneumoniae* strain 65-2161 attached to glass in 3-ounce bottles were obtained under similar conditions but without phase-contrast optics.

Lipid analysis. Cholesterol levels were determined by the method of Rudel and Morris (14), a colorimetric assay that employs the *o*-phthalaldehyde reagent of Zlatkis and Zak (24). Various serum fractions and their components were subjected to lipid extraction according to the method of Folch et al. (6). Before further analysis, each lipid extract was chromatographed on Sephadex G-25 to remove the nonlipid contaminants (13). The extracts were dried and maintained in a desiccator in vacuo. Large solvent volumes were reduced under vacuum in a rotary evaporator (Buchler Instruments Div., Nuclear-Chicago Corp., Ft. Lee, N.J.); small volumes were lessened in a warm metal block under a stream of nitrogen. Before addition to culture medium, lipid extracts were dissolved in propylene glycol at 37°C and brought to the original BSF volume with sterile distilled water. The final concentration of propylene glycol in the suspension was 2%. Three percent of this suspension was added to SSR_2 as a BSF substitute.

Chloroform, acetone, and methanol were purchased already glass distilled from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.) or, when purchased from other sources, redistilled shortly before use. All other solvents were reagent grade. Lipid extracts were separated into neutral lipid, glycolipid, and phospholipid classes on a 100–200 mesh silicic acid column (Clarkson Chemical Co., Inc., Williamsport, Pa.) with chloroform, acetone, and methanol, consecutively, as eluates (2). The percentage of the total lipids present in each class was determined by weight.

RESULTS

Nutrient value and growth-promoting activity of BSF lots. Of the four lots of BSF tested, BSF-B contained the highest growth-promoting activity for *M. pneumoniae* strain 65-2161, based primarily on the density of mycoplasma colonies noted with this BSF in the culture medium. Use of BSF-C produced a sparse quantity of mycoplasmas, and there were many areas of the glass surface that did not contain organisms. However, in contrast to previously published results (20), an entirely uniform growth, devoid of clumps of organisms, was not obtained with any of the BSF lots. In terms of GAM protein, different lots of BSF varied considerably in their ability to promote growth. Our best quality lot was BSF-B, and our worst was BSF-C (Table 1). Use of BSF-B yielded about double the GAM protein values of the other three BSF lots.

Table 1 also shows the protein and cholesterol levels of the BSF lots, as well as a comparison of the bands obtained by electrophoresis. The high-growth-promoting lot, BSF-B, had the highest protein content. The BSF-B also contained a greater quantity of cholesterol than any other BSF, approximately double that of BSF-A and BSF-C, and over one-third higher than BSF-D. Also, BSF-B contained a strong alpha band, as contrasted to a fainter band found in BSF-C and none in BSF-A. No band corresponding to LDL was found in any lot.

Because of the superior growth-promoting ability of BSF-B, we established this lot as a standard for comparison with all other lots; BSF-C was considered a substandard lot useful for testing effects of supplements.

Nutrient and growth-promoting activity of BSF components. The third and fourth

TABLE 1. *Characteristics of four lots of BSF compared with their ability to support growth*

BSF	Glass-adherent mycoplasmas (μg of protein)[a]		Electrophoretic bands[b]	Cholesterol content (mg/100 ml)	Protein content (mg/ml)
	G-250	Lowry			
BSF lot					
A	144 ± 9;153 ± 7[c]	ND[d]	None	59	70.2
B	317 ± 7	810 ± 58	Alpha, trace pre-beta	171	84.2
C	125 ± 12	ND	Alpha	88	60.6
D	183 ± 5;162 ± 12[c]	ND	ND	113	67.5
BSF component[e]					
A-3	ND	929 ± 10	None	125	1.2
A-4	ND	233 ± 13	None	<10	0.9
B-3	ND	990 ± 17	Trace, alpha	161	0.6
B-4	ND	886 + 68	Alpha	162	2.3
C-3	5	ND	None	<10	<0.1
C-4	147 ± 6	ND	Alpha	110	2.3

[a] Growth of *M. pneumoniae* strain 65-2161 as measured by either the G-250 or Lowry assay (± one standard error of the mean). Lowry determinations yielded values about three times higher than those with G-250 (for comparison, see BSF-B). Values represent the mean of triplicate cultures. Each BSF or BSF component was used at 3% final concentration in the medium.

[b] Bands observed after lipoprotein electrophoresis on agarose strips.

[c] Data from other experiments.

[d] ND, Not determined.

[e] Components were obtained at different densities through ultracentrifugation (7). Media containing BSF components were supplemented with 0.4% BSA.

ultracentrifugation fractions corresponding to the LDL and HDL of three BSF lots contained very little protein (Table 1). Since the separation procedure was designed to eliminate albumin from these fractions, this protein deficit was remedied by the addition, along with each component, of 0.4% BSA to the medium. Further studies indicated that greater concentrations of BSA did not result in increased growth, whereas there was very little growth when the components were used without additional BSA (data not shown).

Growth-promoting activity for *M. pneumoniae* strain 65-2161 was markedly different for components from each BSF lot (Table 1). With BSF-A, as reported previously (22), component 3 yielded about three times more GAM protein than did component 4. However, with BSF-B, component 4 was as active as component 3; with either component, growth equalled that of intact BSF-B. In contrast with results with both of these lots, the third component of BSF-C contained virtually no activity.

There was no evidence of intact beta lipoproteins (LDL) in component 3 of any of the BSF lots. Alpha lipoproteins (HDL) were present in component 4 of both BSF-B and BSF-C, and both of these components promoted growth of *M. pneumoniae*. However, high growth levels were also obtained from BSF-A component 3 that contained no detectable lipoprotein and from a BSF-B component that contained only a trace amount of alpha lipoprotein. Thus, we could not attribute the growth-promoting ability of BSF ultracentrifugation components to the presence of any particular lipoprotein.

The cholesterol level of BSF-A component 3 far exceeded that of component 4; the cholesterol levels of BSF-B components 3 and 4 are almost identical. However, in BSF-C component 4, there was no measurable cholesterol.

Our data indicated that the higher the cholesterol level of a component, the greater its ability to support the growth of *M. pneumoniae*. The higher cholesterol values obtained with the components as compared with unfractionated BSF was presumably due to a concentration effect resulting from the centrifugation process.

Lipid analyses. Whereas BSF-A and BSF-C contained similar quantities of total lipids, BSF-B possessed almost twice that amount (Table 2). We questioned whether the high lipid content of BSF-B was due to a concentration of any one lipid class. BSF-B contained more than twice the phospholipid, and just under twice the neutral lipid, of the other two lots. Although the glycolipid differences were even greater, the quantities obtained were small. We lost 3 to 4% of the lipid samples during our chromatographic analysis; therefore, these differences may not be

TABLE 2. *Lipid composition of three lots of BSF*[a]

BSF lot	Lipid (mg/ml)	Neutral lipids		Glycolipids		Phospholipids	
		mg/ml	%	mg/ml	%	mg/ml	%
A	1.97	1.42	75	0.03	1	0.46	24
B	3.88	2.50	65	0.24	6	1.02	28
C	1.86	1.31	73	0.09	5	0.40	22

[a] Values are averages of two experiments. Lipid extracts were chromatographed on a silicic acid column. Neutral lipids were eluted with chloroform, glycolipids were eluted with acetone, and phospholipids were eluted with methanol, in that order. Each fraction was dried to a constant weight.

significant. Also, for all the BSFs, the percentages of each lipid class per total lipid are roughly equivalent.

Effect of BSF concentration on the growth of *M. pneumoniae*. We studied the effect of increasing the final concentrations of our standard BSF-B and of substandard BSF-C, above the usual 3% concentration in the culture medium. Growth of strain 65-2161 with BSF-C reached an optimum when it was used at final concentrations of 12 to 15% in the medium (Fig. 1A). With BSF-B (Fig. 1B), the standard lot, there were minimal differences in growth levels when concentrations ranged from 3 to 9%, although the higher concentrations achieved maximum growth in a shorter time. At 12%, BSF-B was inhibitory.

Medium supplemented with cholesterol for growth of *M. pneumoniae*. To specifically measure the effect of cholesterol on mycoplasma growth, we added 1, 3, and 5 mg of a water-based cholesterol suspension per 100 ml to BSF-C at its usual 3% concentration. Normally, BSF-C supplies about 2.6 mg of cholesterol per 100 ml to the medium (refer to Table 1; 3% of 88 mg/100 ml); the addition of 3 mg/100 ml would approximate the level of 3% BSF-B (5.2 mg/100 ml). We also tested cholesterol in the absence of BSF-C by using 0.5% BSA as a protein source. As a comparison, BSA at the same concentration was included with BSF-C plus 5 mg of cholesterol per 100 ml.

As expected, BSF-C alone resulted in poor growth of strain 65-2161 (Fig. 2), less than half that obtained with BSF-B. Growth was significantly increased by addition of cholesterol. When albumin was incorporated along with the cholesterol, the GAM protein level reached that of BSF-B. Surprisingly, when BSF was omitted and replaced with only 5 mg of cholesterol per 100 ml plus albumin, *M. pneumoniae* reached a growth level as high as one-half that observed when we used BSF-B (Fig. 2).

Similar effects were seen with strains CL-8 and FH. Increased concentrations of cholesterol in conjunction with BSF-C elevated GAM protein slightly above that of BSF-C alone. With addition of albumin to BSF-C plus 5 mg of cholesterol per 100 ml, growth in both strains was significantly higher than with BSF-C alone and equalled that of BSF-B. Growth levels at 50% of that of the best quality serum fraction were achieved with both strains when serum was omitted and only cholesterol and albumin were used.

Results for BSF-D (not shown), which had a cholesterol level well below that of BSF-B (Table 1), were similar to those obtained with BSF-C. With all three strains of *M. pneumoniae*, BSF-D produced only one-half the growth levels of BSF-B. The addition of cholesterol resulted

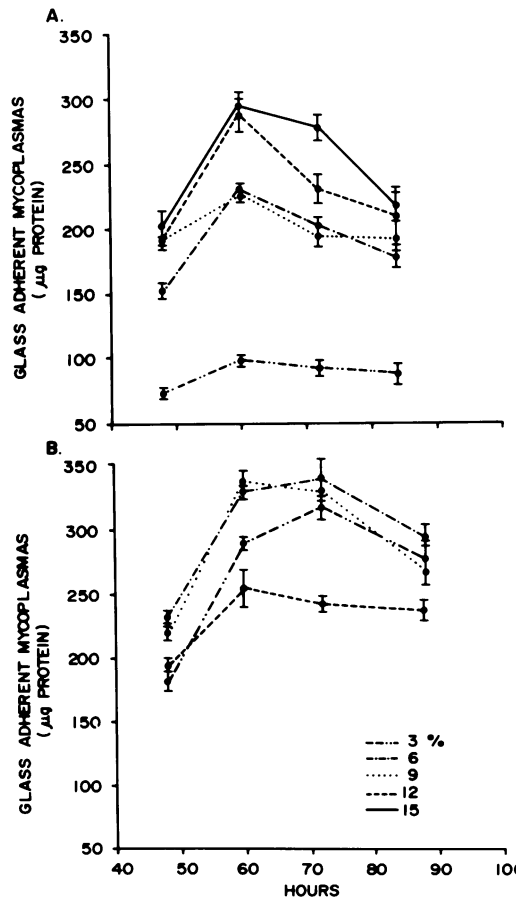

FIG. 1. *Growth of M. pneumoniae strain 65-2161 in SSR_2 broth containing two different lots of BSF. (A) BSF-C; (B) BSF-B. Each point represents the mean of triplicate cultures ± one standard error of the mean.*

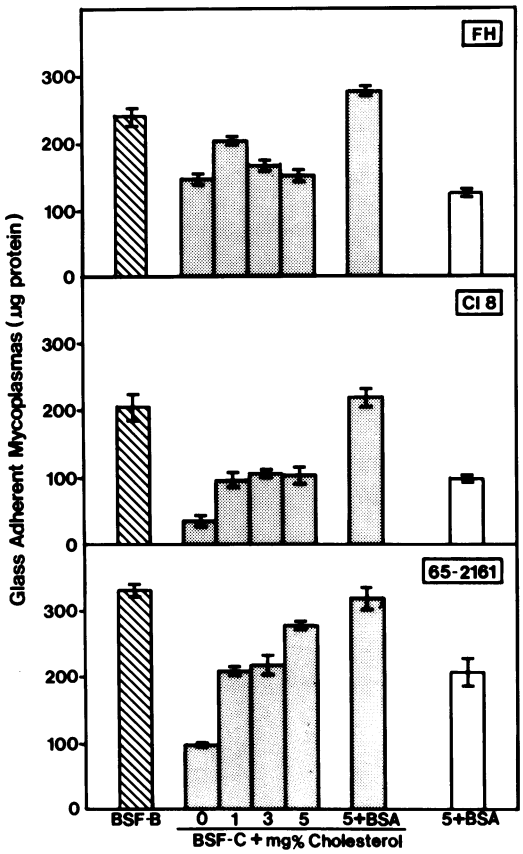

FIG. 2. *Growth of three strains of M. pneumoniae in medium supplemented with cholesterol. Striped bars indicate cultures grown in 3% BSF-B; solid bars indicate growth in 3% BSF-C plus cholesterol at the concentrations indicated. Open bars show cultures grown in 5 mg of cholesterol per 100 ml and BSA in the absence of BSF. When added, BSA was at 0.4% final concentration. Each bar represents the mean of triplicate cultures ± one standard error of the mean.*

in higher GAM protein values, but the maximum value was achieved with inclusion of BSA. With use of both cholesterol and albumin, the GAM protein values were as good or better than those with BSF-B alone.

Effect of cholesterol on GAM appearance. Various levels of cholesterol also produced qualitative differences in GAM. In Fig. 3A, the poor quality of BSF-C at 3% is demonstrated by the sparse growth of organisms on the glass surface. When concentrations of BSF-B, BSF-C, or BSF-C plus cholesterol were adjusted to supply similar cholesterol levels in the medium, the resulting GAM protein values for strain 65-2161 were also similar. However, using BSF-C plus cholesterol, we consistently obtained a more uniform cell sheet. Although high concentrations of BSF-B and BSF-C (Fig. 3B and C) resulted in denser growth, the formation of many isolated mycoplasma colonies was evident. When cholesterol was added to BSF-C (Fig. 3D), the GAM was not only thicker and more uniform than that with BSF-C alone, but also contained fewer isolated colonies. Because of the dense, more consistent appearance of the GAM when cholesterol was added to the medium, this method of attaining optimum cholesterol levels appears to be preferable to increasing the BSF concentration.

Cholesterol as an agar medium supplement for *M. pneumoniae*. We confirmed the importance of the cholesterol level of BSF by using solid medium (Fig. 4). All plates received identical inocula of *M. pneumoniae* strain 65-2161 and were incubated under identical conditions. Figure 4A through F shows representative mycoplasma colonies grown in medium containing three different lots of BSF at 3% concentration, some with added cholesterol and BSA. Consistent with the broth results, BSF-C (Fig. 4A) was clearly inferior in growth-promoting activity to BSF-B (Fig. 4B). More colonies were observed when BSF-B was used; they were larger and their centers appeared to be more distinct. These effects were consistent over the entire surface of the agar.

The appearance of many colonies in a small and irregular form on agar medium containing BSF-B probably resulted from crowding. A concentrated inoculum had to be used to produce colonies on agar containing substandard BSF. When we diluted the inoculum and repeated the experiment, we observed the typical "fried-egg" morphology of colonies on plates containing BSF-B, in greater numbers than those seen on agar with BSF-C.

A poor-quality BSF could replace a high-quality BSF if cholesterol was also incorporated into the agar (Fig. 4C). The colonies were comparable to those grown on BSF-B and significantly larger and greater in number than those seen on BSF-C alone (Fig. 4A).

The addition of BSA to BSF-C plus cholesterol (Fig. 4D) did not result in any marked change. However, without a colony count, only dramatic differences, as seen when Fig. 4A is compared with Fig. 4B and C, would be observed. Since crowding hindered colony counting, we diluted the inoculum and maintained identical culture conditions. When these colony counts were compared, there were approximately double the number of colonies on plates with added BSA.

We also added cholesterol and BSA to plates containing BSF-D (Fig. 4F). There were a

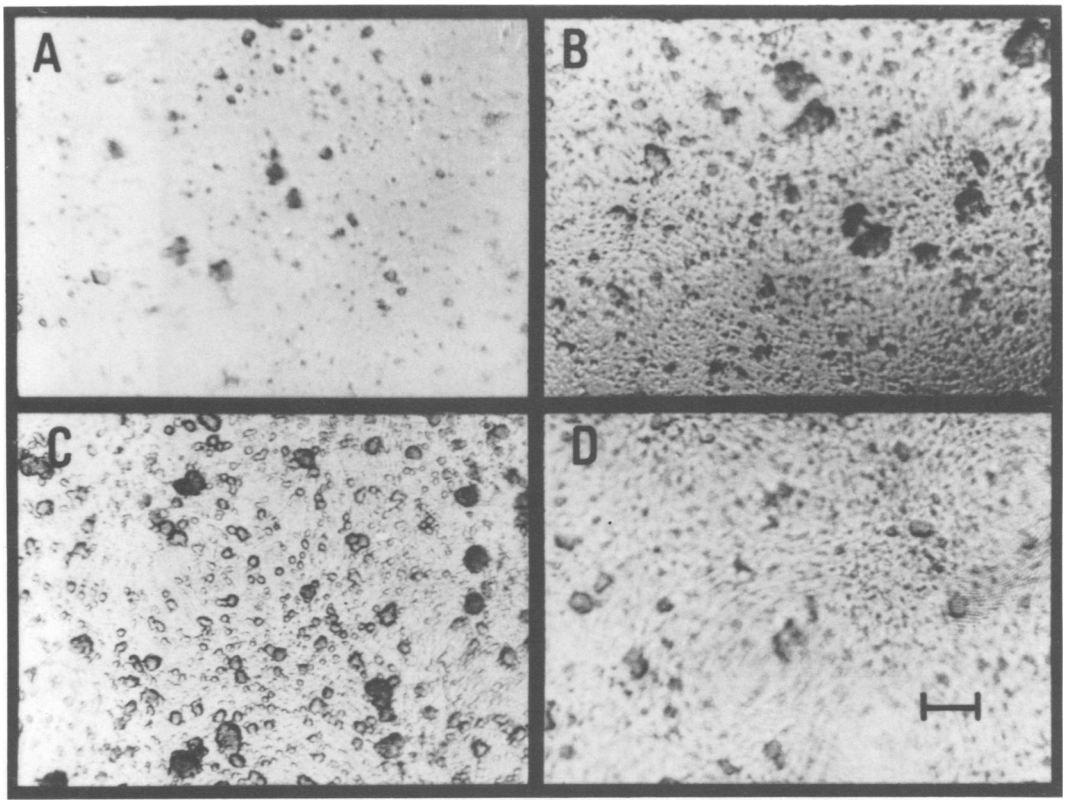

FIG. 3. *M. pneumoniae strain 65-2161 grown as glass-adherent organisms. Variation of the concentration and source of BSF in conditions B through D resulted in equivalent cholesterol levels (10 mg/100 ml) in the final medium. Cultures were grown in SSR_2 broth containing (A) 3% BSF-C, (B) 6% BSF-B, (C) 12% BSF-C, and (D) 3% BSF-C plus 7.5 mg of cholesterol per 100 ml and 0.4% BSA. Bar, 0.1 mm.*

greater number of colonies and more with well-developed centers on this medium as compared with agar containing BSF-D without cholesterol or BSA (Fig. 4E). The results are similar to those described for BSF-C. Although the colonies seen in Fig. 4F do not appear larger than some on Fig. 4E, again this is probably a crowding effect.

In addition, we incorporated increased concentrations of BSF into agar medium. Both BSF-C and BSF-B levels were raised so that the culture medium would contain approximately 10 mg of cholesterol per 100 ml (Fig. 4G and H). In both cases, colony size and numbers were greater than those seen when the standard 3% BSF level was used (Fig. 4A and B). However, on agar containing BSF-B, a greater number of colonies attained a larger size and possessed more distinct centers than those on BSF-C.

DISCUSSION

The growth-promoting activity of serum for mycoplasmas has been ascribed to various lipoproteins. We confirmed the earlier report of Washburn and co-workers (22), who showed that intact serum lipoproteins were not necessary to sustain the growth of *M. pneumoniae* in SSR_2 medium. We also observed that high growth-promoting activity corresponded to high cholesterol content not only for the BSF ultracentrifugation components, but also for each of four commercial lots of BSF.

The variability among commercial lots of BSF was eliminated by increasing their cholesterol content to an optimum level, with final concentrations of 5 to 10 mg/100 ml producing the best results. These levels could be achieved either by increasing the amount of BSF in the medium or by adding a cholesterol suspension to medium containing BSF. When the concentrations of BSF-B and BSF-C resulted in 10 mg of cholesterol per 100 ml (by calculation from Table 1; for BSF-B, 6% of 171 mg/100 ml, and for BSF-C, 12% of 88 mg/100 ml), we not only reached maximum growth values for *M. pneumoniae*, but also did so in a shorter time than when lower

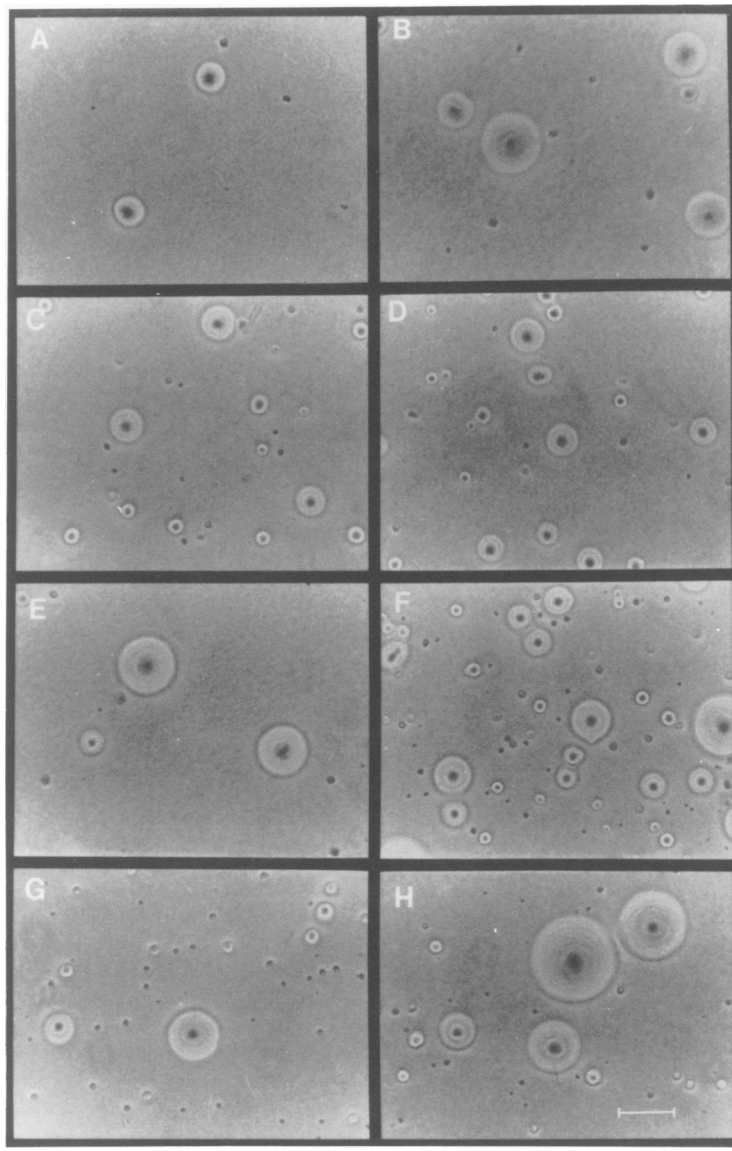

FIG. 4. *Effect of cholesterol addition on colony formation on M. pneumoniae strain 65-2161. Plates A through F contain BSF at 3%. Mycoplasmas were grown on agar medium supplemented with (A) BSF-C, (B) BSF-B, (C) BSF-C plus 5 mg of cholesterol per 100 ml, (D) the preceding condition with 0.4% BSA, (E) BSF-D, (F) BSF-D plus 5 mg of cholesterol per 100 ml and 0.4% BSA, (G) 12% BSF-C, and (H) 6% BSF-B. Bar, 0.1 mm.*

concentrations of BSF were used (Fig. 1).

The addition of cholesterol, along with albumin, to the medium containing substandard serum fraction was sufficient to raise growth levels to those obtained with our highest quality BSF. This effect was demonstrated for three strains of *M. pneumoniae* in both agar and broth. Growth stimulation under these conditions indicated that improvements previously noted when higher BSF concentrations were used resulted from higher cholesterol content rather than from some other serum factor.

For growth of *M. pneumoniae*, the addition of a cholesterol suspension to the medium had multiple effects. The glass-adherent cell mass cultured in media supplemented with cholesterol was denser and more uniform than that grown in media containing increased BSF concentrations. This result was not due to the inclusion of albumin, since these differences in the

GAM were observed in the presence of supplemental cholesterol even when BSA was absent (unpublished data). Also, we have not noticed any change in the appearance of GAM when BSA alone was added to BSF.

The success obtained with substandard BSF augmented with cholesterol as a substitute for good BSF was demonstrated quantitatively in broth and on solid media. The high number of *M. pneumoniae* colonies recovered on plates containing added cholesterol suggests that such a medium might be useful for the primary isolation of *M. pneumoniae* from clinical specimens. Testing various sources of serum for use in mycoplasma media is not routine in most clinical laboratories. An adjustment of the cholesterol and albumin levels of any serum product could be a simple and convenient method for improving isolations.

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LITERATURE CITED

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.
2. **Carroll, K. K., and B. Serdarvich.** 1967. Column chromatography of neutral glycerides and fatty acids, p. 205–237. *In* G. V. Marinetti (ed.), Lipid chromatographic analysis. Marcel Dekker, Inc., New York.
3. **DiVecchia, L., and N. L. Somerson.** 1973. Tetrazolium reduction as a measure of metabolic activity for glass-adherent *Mycoplasma pneumoniae*. Appl. Microbiol. **26:**298–302.
4. **Edward, D. G. ff.** 1971. Determination of sterol requirement for *Mycoplasmatales*. J. Gen. Microbiol. **69:**205–210.
5. **Edward, D. G. ff., and W. A. Fitzgerald.** 1951. Cholesterol in the growth of organisms of the pleuropneumonia-like group. J. Gen. Microbiol. **5:**576–586.
6. **Folch, J., M. Lees, and G. H. Stanley.** 1957. A simple method for the isolation and purification of total lipides from animal tissue. J. Biol. Chem. **226:**497–509.
7. **Hatch, F. T., and R. S. Lees.** 1968. Practical methods for plasma lipoprotein analysis. Adv. Lipid Res. **6:**33–36.
8. **Hayflick, L.** 1965. Tissue cultures and mycoplasmas. Tex. Rep. Biol. Med. **23:**285–296.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
10. **Noble, R. P.** 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. J. Lipid Res. **9:**693–700.
11. **Pollack, J. D., D. S. Clark, and N. L. Somerson.** 1971. Four-directional development thin-layer chromatography of lipids using trimethyl borate. J. Lipid Res. **12:** 563–569.
12. **Razin, S., and J. G. Tully.** 1970. Cholesterol requirement of mycoplasmas. J. Bacteriol. **102:**306–310.
13. **Rouser, G., G. Kritchevsky, and A. Yamamoto.** 1967. Column chromatographic and associated procedures for separation and determination of phosphotides and glycolipids, p. 99–162. *In* G. V. Marinetti (ed.), Lipid chromatographic analysis. Marcel Dekker, Inc., New York.
14. **Rudel, L. L., and M. D. Morris.** 1973. Determination of cholesterol using o-phthalaldehyde. J. Lipid Res. **14:** 364–366.
15. **Slutzky, G. M., S. Razin, and I. Kahane.** 1976. Serum lipoproteins as cholesterol donors to mycoplasma membranes. Biochem. Biophys. Res. Commun. **68:**529–536.
16. **Slutzky, G. M., S. Razin, I. Kahane, and S. Eisenberg.** 1977. Cholesterol transfer from serum lipoproteins to mycoplasma membranes. Biochemistry **16:**5158–5163.
17. **Smith, P. F., and H. E. Morton.** 1951. The separation and characterization of the growth factor in serum and ascitic fluid which is required by certain pleuropneumonia-like organisms. J. Bacteriol. **61:**395–405.
18. **Smith, P. F., and H. E. Morton.** 1952. Further characterization of the protein factor required by certain pleuropneumonia-like organisms for growth *in vitro*. Arch. Biochem. Biophys. **38:**23–28.
19. **Somerson, N. L., W. D. James, B. E. Walls, and R. M. Chanock.** 1967. Growth of *Mycoplasma pneumoniae* on glass surfaces. Ann. N.Y. Acad. Sci. **143:**384–389.
20. **Somerson, N. L., L. B. Senterfit, and V. V. Hamparian.** 1973. Development of a *Mycoplasma pneumoniae* vaccine. Ann. N.Y. Acad. Sci. **225:**425–435.
21. **Spector, T.** 1978. Refinement of the Coomassie Blue method of protein quantitation. Anal. Biochem. **86:**143–146.
22. **Washburn, L. R., J. H. Hughes, and N. L. Somerson.** 1978. Mycoplasma growth factors in bovine serum fraction. J. Bacteriol. **135:**818–827.
23. **Washburn, L. R., and N. L. Somerson.** 1979. Lipoproteins as substitutes for serum in mycoplasma culture medium. J. Clin. Microbiol. **10:**586–589.
24. **Zlatkis, A., and B. Zak.** 1969. Study of a new cholesterol reagent. Anal. Biochem. **29:**143–148.